Activation of liver macrophages to tumor cytotoxicity with liposome-encapsulated muramyl dipeptide

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SUMMARY AND CONCLUDING REMARKS

The major experimental results presented in this thesis demonstrate that rat liver macrophages can be activated *in vitro* to a tumor cytotoxic state with both free and liposome-encapsulated muramyl dipeptide (MDP) and that upon systemic administration of liposomal MDP a significant reduction in the development of hepatic metastases in a murine liver metastasizing tumor model can be obtained. In addition, a number of detailed studies was performed, specifying optimal experimental conditions and attempting to throw some light on the mechanism of macrophage activation. The major findings of this work are summarized and briefly discussed in the following paragraphs.

In the Introduction (**chapter 1**), the position of macrophage activation within the framework of the immunological system is depicted; the treatment modalities for liver metastases are summarized and the immunomodulator MDP and liposomes as drug carriers are briefly introduced.

Chapter 2 shows the capacity of rat liver macrophages to lyse cocultured tumor cells and to inhibit their proliferation after exposure of the macrophages *in vitro* to MDP, lipopolysaccharide (LPS) or liposome-encapsulated MDP. Consistent with other studies concerning combinations of immunomodulating agents, we found a synergistic effect of MDP and LPS on the tumoricidal potency of the macrophages. Encapsulation of MDP within multilamellar liposomes was shown to strongly reduce the amount of MDP needed for optimal activation and furthermore to increase the maximally obtainable level of tumor cell lysis. Additionally, we observed that a higher load of liposomal lipid carrying MDP into the macrophages induced a higher level of tumor cell lysis. This observation led us to the preliminary hypothesis that a sustained intracellular release of MDP was beneficial for the activation of liver macrophages to a tumoricidal state. A number of experiments designed to verify this hypothesis is described in chapter 3.

Since it was known that the composition of the liposomal lipids influences the intralysosomal degradation of liposomes by phospholipases, we studied the possibility of manipulating the extent and duration of macrophage activation by applying different liposomal lipid compositions thus supposedly manipulating the rate of intracellular release of MDP (**chapter 3**). It was observed however that neither the extent nor the duration of tumor cytotoxicity could be altered in this way. We now presume that a very low dose of intracellular MDP suffices for the activation of macrophages to a tumoricidal state and that, moreover, the macrophages become (temporarily) refractory to activation with MDP. Thus, a major fraction of MDP released intracellularly from the "heavily loaded" liposomes as used in chapter 2 would not be necessary for efficient activation of the cells. It should be noted, however, that encapsulation of MDP within liposomes enhanced both the extent and duration of the activated state

compared to free MDP (chapter 6). This finding can presumably be ascribed to a higher uptake by the macrophages of liposomal compared to free MDP, as shown by others using ^{3}H-labeled MDP and resident peritoneal macrophages or monocytes. In addition, a more efficient access of liposomal MDP to intracellular sites in the macrophages as compared to the free drug can not be excluded at this moment.

In **chapter 3** some data are presented on the cytolytic and cytostatic effect of activated liver macrophages on different tumor- and normal cell lines. It appeared that although activated macrophages do not lyse normal cells they do inhibit proliferation of these cells *in vitro*. The significance of this effect in an *in vivo* situation has not been determined although during *in vivo* experiments we never observed any morphological effects on cells adjacent to liver macrophages, e.g. hepatocytes, upon repeated injections of liposomal MDP (not shown). Various tumor cell lines differed in susceptibility to the cytolytic effect but did not differ in susceptibility to the cytostatic effect.

That experimental culture conditions may influence the results was shown by the observation that the level of tumor cell lysis decreased with a decrease in the density of the macrophage monolayer culture. The macrophage to tumor cell ratio on the other hand did not, within the range studied, affect the induced tumor cell lysis.

The time of coculture also determines the outcome of the experiments: maximal proliferation inhibition of tumor cells was obtained after 24 h in coculture while maximal tumor cell lysis required 48 h of coculture.

The observations described in chapter 2 on the synergistic effect of MDP and LPS might be exploited to increase the *in vivo* therapeutic effect of MDP. Since, however, systemic administration of LPS would induce severe toxic side effects, we studied whether this could be circumvented by incorporating LPS or lipid A, i.e. the immunoactive component of LPS, within liposomes. However, experiments, as described in **chapter 4**, showed that in contrast to MDP, the incorporation of LPS and lipid A reduced the activating potency significantly. Since liposomes, after uptake by macrophages, are degraded by lysosomal phospholipases, we presumed that both, LPS and lipid A, might be inactivated by lysosomal enzyme activities. This became more likely after our observation that incubation of LPS and lipid A with a lysosomal fraction isolated from rat liver inactivated these agents in contrast to MDP which retained full activity following the same treatment.

In **chapters 5 and 6** the relevance of the heterogeneity of the rat liver macrophage population with respect to phagocytic and tumoricidal activity is described. Liver macrophages were separated into 3 or 5 subpopulations on the basis of cell size by means of centrifugal elutriation. The large-size macrophages were both *in vitro* and *in vivo* the most potent in the phagocytosis of liposomes whereas the small- to intermediate-size macrophages were the most potent in tumoricidal activity. With respect to the tumoricidal activity it was furthermore shown that during *in vitro* culture the potency of large-size cells to become tumoricidal decreased earlier than that of the smaller cells and the tumoricidal activity induced with MDP was maintained longer in the smaller cells compared to the larger cells. A second exposure of the macrophages to

LPS and MDP, 48 h after the first exposure to the same agent, showed a considerable reduction in the responsiveness in the small- to intermediate size cells and a complete lack of response in the large cells.
The intravenous adminstration of liposomal MDP resulted in equal levels of tumoricidal activity of all subpopulations. However, the cell populations isolated from these "activated" livers are not identical to the liver macrophage subpopulations isolated from unstimulated rats as is described in the next chapter, i.e. **chapter 7**. By employing two monoclonal antibodies specific for cells belonging to the mononuclear phagocyte system, we found a doubling of the hepatic macrophage population within 24 h after administration of a single dose of liposomal MDP. The numerical increase was a result of, on the one hand, influx of "undifferentiated" macrophages and, on the other, proliferation of both resident and recruited macrophages. Proliferation of macrophages was demonstrated with a monoclonal antibody specific for bromodesoxyuridine which after intravenous administration is incorporated in the DNA of replicating cells.
The macrophages isolated from an "activated" liver presumably differ in quantity and differentiation state. It is postulated that the expansion of the liver macrophage population may be essential for the effectiveness of this kind of immunotherapy. By investigating a number of structural and functional parameters of the macrophage population during *in vivo* activation into more detail, this hypothesis may be tested. The data thus obtained may help to design optimal therapeutic protocols for immunotherapy of liver metastases.

Finally, the applicability of liposomal MDP treatment in a murine liver metastasizing tumor model is described (**chapter 8**). For these studies we applied a murine model, which involves the inoculation of colon adenocarcinoma cells into the spleen of syngeneic mice, resulting in liver metastases in 100% of the animals. Liposomal MDP treatment strongly reduced the development of hepatic metastases and prolonged the survival time of the mice significantly. It was observed that an early initiation of therapy, i.e. 2 days prior to tumor cell inoculation resulted in a substantially better therapeutic effect compared to initiation one day after inoculation.

In our view, this immunotherapeutic approach can potentially be applied as a single treatment to treat micrometastases or in combination with other treatment modalities to attack existing metastases. The first approach could be used for cancer patients having a high risk of metastatic disease, without detectable metastases yet, the second approach might be applied in patients with metastases in the liver. By combining the potency of surgery and chemo- or radiotherapy to eliminate the bulk of the tumor mass in the liver, activated macrophages could subsequently destroy the residual tumor cells. The feasibility of combining immunotherapy with chemo- or radiotherapy will need thorough investigation however; for example one of the pitfalls of such a combination might be that chemo- or radiotherapy could interfere with the expansion of the liver macrophage population which, as we pointed out, is likely to be pertinent for effective immunotherapy. Continuing research along the lines set out in this thesis will therefore be required to design optimal therapeutic protocols for effective treatment of liver metastases.